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A purified retroviral envelope polypeptide, isolated nucleic acids encoding said polypeptide, vectors and use thereof

Field of invention

5 The present invention relates to a purified retroviral envelope polypeptide, which is capable of mediating infection of a cell, by use of the polytropic/xenotropic receptor encoded by the Rmc1 locus from a NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of a cell by use of a human polytropic/xenotropic receptor encoded by the human RMC1 locus

10 In a presently preferred embodiment, the present invention relates to an envelope protein from the Murine Leukaemia Virus (MLV) strain SL3-2, which is capable of infecting murine cells through usage of the polytropic receptor encoded by the Rmc1 locus, but lacks the ability of infecting human cells expressing the corresponding xenotropic receptor encoded by the RMC1 locus

15 Background of the invention

Murine Leukaemia viruses are a family of simple retroviruses isolated from laboratory mice. Retroviruses carry their genomes as two copies of a single RNA molecule and the simplest retroviruses contain the *gag*, *pro*, *pol* and *env* genes. These genes are found in the same order in all known retroviruses, reflecting the phylogenetic relationship of
20 retroviruses

The first step in the replication cycle of a retrovirus is its entry into a host cell. The envelope protein (*env*) is responsible for binding of the retrovirus to a specific cell surface receptor. A retroviral receptor is a membrane integral protein in the plasma membrane of the host cell and as such has a function unrelated to virus infection. From the non-
25 retroviruses capable of pseudotyping, retroviral envelopes that use non-protein receptors are known, e.g. the vesicular stomatitis virus

Retroviruses can be thought of as a protein-package comprising RNA wrapped in a lipid membrane that contains glycoproteins. The lipid bi-layer is derived from the cell membrane after budding and is thought to be associated with a viral gene product, a
30 peripheral membrane protein called Matrix (MA). Traversing through the lipid bi-layer is another viral gene product, the envelope protein, which consists of two subunits: the transmembrane (TM) and the surface unit (SU). The function of the envelope protein is binding of the virus to its target cell and mediating fusion of the viral and cellular membranes

35 Interference studies have defined several different groups of MLVs depending on their receptor usage. Ecotropic viruses utilise the mCAT-1 receptor and are unable to infect non-murine cells. The amphotropic virus uses another receptor and are able to infect cells from a wide variety of organisms including humans. The 10A1 virus is able to bind to two different receptors, both the amphotropic Pit-2, but also the homologous Pit-1 receptor,

and has a similarly wide tropism. Finally, the xenotropic viruses utilise the same receptor as the polytropic viruses, but are unable to infect murine cells

The SL3-2 murine leukaemia virus was isolated from a mouse leukaemia cell line (Pedersen et al, 1981). This virus has a host range similar to mouse ecotropic viruses in that it replicates in mouse cells but not in cell lines such as mink and dog cells. Its ability to infect human cells has not previously been investigated

By RNA oligonucleotide fingerprinting and by receptor interference studies on mouse cells SL3-2 have been related to mouse MCF viruses. However, it does not have the polytropic species host range characteristic of MCF viruses

- 10 The above mentioned observations were done using biological isolate, which have not previously been cloned. The SL3-2 virus has been given little attention over the past 15 years

- 15 Ecotropic and amphotropic MLVs have been widely used as research tools. Ecotropic viruses are usually chosen because of safety concerns, while the amphotropic viruses have the ability to infect human cells. Different packaging cell lines that express the ecotropic or amphotropic envelopes have been designed to fulfil these different requirements

- 20 Retroviral integration can activate genes in the vicinity of the integration site. In this way, retroviruses have been used to identify oncogenes since activation of these genes result in tumour growth. In much the same way the integration of a provirus can disrupt the expression of genes, hence inactivation of a tumour suppressor gene may contribute to tumour formation. A high number of integrations are desirable in such studies since not all integrations result in tumour generation and multiple hits are required. Very few integration events are expected to be near oncogene or tumour suppressor genes. Tumour formation might also involve multiple gene regulations

- 25 Retroviral infections usually result in a single integration event since the envelope protein blocks receptors on an infected cell. This is the basis of the superinfection resistance (also called interference) phenomenon in which a virus-infected cell shows resistance to superinfection by viruses, which utilise the same receptor for entry. Thus, use of viruses with different receptor usage increases the number of integration events. Entry by different receptors may even provide access to retroviral disease induction in different mouse tissues

- 35 The integration mechanism of retroviruses can be used to introduce any DNA sequence into a host genome, if the appropriate *cis* elements of the retroviral genome is maintained in the transducing vector and the DNA sequence can be encompassed in the vector (less than 9000 bp). Therefore retroviral vectors are attractive tools for gene therapy. Most simple retroviral receptors are found on many different cell types of the same species. That is why vector systems utilising wild type envelopes from simple retroviruses cannot be used to introduce genes in a selective manner into specific cells/tissues. Retargeting envelope proteins remains an elusive goal

While many attempts have been done to change the tropism of viruses by introducing novel binding domains such as single chain antibodies into envelope proteins, most have been unsuccessful. The chimeric envelopes usually obtain the ability to bind to the

- 5 intended target, but lose their fusion activity. In some cases expression of the wild type envelope is necessary to achieve any infection of the target cells. Ecotropic envelopes have been used in most of such studies since they lack the ability to infect human cells. Chimeric envelope based on an ecotropic envelope is unlikely to cause secondary, unintended infections.

- 10 Within the well-characterised group of gamma-retroviruses, other envelopes that do not infect human cells have not been available. So far, there has not been any explanation for the species tropism of gamma-retroviruses. Any research into this area that results in methods for alterations of species tropism may have important consequences for designing vectors as tools for human gene therapy.

Detailed description of the invention

The present invention relates to a new purified retroviral envelope polypeptide originating from the Murine Leukaemia Virus (MLV) SL3-2. As shown by the present inventors, this new polypeptide is capable of mediating infection of a cell, by use of the

- 5 polytropic/xenotropic receptor encoded by the Rmc1 locus of the NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of a cell by use of a human polytropic/xenotropic receptor encoded by the human RCM1 locus

- The present invention also relates to a new defined region within said polypeptide named VR3. Surprisingly, changing specific amino acids within the VR3 region can alter the host
10 tropism of SL3-2. The present inventors has pin-pointed exactly which amino acid that is essential for this host tropism shift and thus relates to substitutions of a amino acid within the VR3 region

- The present invention further relates to cells, capable of displaying, vectors comprising,
15 packaging cells comprising, the envelopes described by the invention. Further, the use of said envelopes for development of gene therapy, gene discovery, and safety aspects are also within the scope of the present invention

The SL3-2 envelope polypeptide

- 20 The SL3-2 envelope polypeptide described in the present application specifically uses the polytropic/xenotropic receptor encoded by the Rmc1 locus of the NIH Swiss inbred NFS/N mouse for entry. Thus, the SL3-2 envelope polypeptide of the present invention is unable to enter a cell by use of the human homolog of said receptor. The polytropic/xenotropic receptor encoded by the Rmc1 locus originally derived from a NIH Swiss inbred NFS/N
25 mouse, can be transferred to any cell by an expression vector comprising said receptor and thereby enabling entry. Thus, one embodiment of the present invention relates to a purified retroviral envelope polypeptide, capable of mediating infection of a cell, by use of the polytropic/xenotropic receptor encoded by the Rmc1 locus isolated from a NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of a cell by use of a
30 human polytropic/xenotropic receptor encoded by the human RMC1 locus

- Another embodiment of the present invention relates to a purified retroviral envelope polypeptide, capable of mediating infection of a cell derived from *Mus musculus* by use of the polytropic/xenotropic receptor encoded by the Rmc1 locus isolated from a NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of a human cell
35 comprising a human polytropic/xenotropic receptor encoded by the human RMC1 locus

A presently preferred embodiment of the present invention relates to a purified murine retroviral envelope polypeptide, capable of mediating infecting of a cell derived from *Mus musculus*, by use of the polytropic/xenotropic receptor encoded by the Rmc1 locus isolated from e.g. a NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of

a human cell comprising a human polytropic/xenotropic receptor encoded by the human RMC1 locus

In the present context the term "unable of mediating infection of a human cell" refers to virus particles comprising the SL3-2 envelope, not capable of infecting human cells, while
 5 capable of infecting mouse NIH -3T3 cells In a laboratory test this term can further be defined as an isolate of virus particles comprising said SL3-2 envelope providing a titer below 10^2 infectious units per ml on the human cells, while at the same time providing a titer above 10^5 infectious units per ml on the murine cells, or as an isolate of viruses comprising said SL3-2 envelope where the difference in titer between the permissive (in
 10 casu laboratory mouse cells) and the non-permissive cells (in casu human cells) is greater than 10^3 infectious units per ml

Fv-1 restriction

Murine cells have several restrictions to retrovirus infection One such restriction is the Fv-1 restriction that operates after binding to the receptor and internalisation of the virus and
 15 before integration of the virus into the host genome This restriction is mediated through an endogenous *gag* like sequence that interacts with the *gag* protein CA in the virus core particle, disabling the normal integration process of the virus A cell can be either Fv-1^{n/n}, Fv-1^{b/b}, Fv-1^{n/b}, or Fv-1^{o/o} The virus can be either N-, B-, or NB-tropic

20 Fv-1 is a co-dominant trait, as Fv-1^{n/b} animals can not be infected by neither N- nor B-tropic viruses, only by NB-tropic viruses Fv-1^{n/n} animals can be infected by N-tropic viruses and vice versa Fv-1^{b/b} can be infected by B-tropic viruses The restriction is not 100%, rather the titer is reduced between 50-1000 fold in a Fv-1 permissive contra a Fv-1 non-permissive cells

25 Almost all or all MCF viruses described are N-tropic in host range (Hartley et al , 1977) meaning that these viruses can infect cells that are Fv-1^{n/n}, but not Fv-1^{b/b}

Human cells have a restriction very similar to this Fv-1 restriction (Towers et al ,
 30 1999)(Aagaard et al , 2002) All human cells tested so far, show a restriction that prevents N-tropic virus infection resulting in a general restriction of human cells to wild type infection of MCF viruses, even if initial attachment and fusion of the virus particle is successful

35 To assay if a given virus can or cannot utilise the human polytropic/xenotropic receptor encoded by the RMC1 locus, the receptor has to be expressed in a cell permissive for N-tropic virus infection or the envelope pseudo-typed with a Gag protein of either B- or NB-tropic virus

40 The SL3-2 envelope described by the present inventors, has been characterised in a mini-virus setting, where the Gag/Pol proteins were provided by the Moloney-MLV This virus is of NB-tropic origin and can thus mediate infection of human cells

As described by the present inventors, the SL3-2 envelope has been cloned from genomic DNA of NIH 3T3 cells infected with SL3-2 by PCR amplification. The amplified PCR fragment was subsequently cloned and sequenced.

Thus, one embodiment of the present invention relates to a purified envelope polypeptide having an amino acid sequence which is at least 94% identical to the amino acid sequence shown in SEQ ID NO 2, or a fragment of said amino acid sequence that is at least 94% identical to the amino acid sequence shown in SEQ ID NO 2, such as 94.5% identical, 95% identical, 95.5% identical, 96% identical, 96.5% identical, 97% identical, 97.5% identical, 98% identical, 98.5% identical, 99% identical, or 99.5% identical.

The similarity between two nucleic acid sequences, or two amino acid sequences, is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology), the higher the percentage, the more similar the two sequences will be.

Methods of alignment of sequences for comparison are well-known in the art. Various programs and alignment algorithms are described and presents a detailed consideration of sequence alignment methods and homology calculations, such as VECTOR NTI.

The NCBI Basic Local Alignment Search Tool (BLAST) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, Md.) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. It can be accessed at <http://www.ncbi.nlm.nih.gov/BLAST/>. A description of how to determine sequence identity using this program is available at http://www.ncbi.nlm.nih.gov/BLAST/blast_help.html.

Homologs of the disclosed polypeptides are typically characterized by possession of at least 94% sequence identity counted over the full length alignment with the disclosed amino acid sequence using the NCBI Basic Blast 2.0, gapped blastp with databases such as the nr or swissprot database. Alternatively, one may manually align the sequences and count the number of identical amino acids. This number divided by the total number of amino acids in your sequence multiplied by 100 results in the percent identity.

In a presently preferred embodiment the envelope polypeptide has the amino acid sequence shown in SEQ ID NO 2.

The SL3-2 envelope shows quite high homology with the envelope protein of MCF-247 polytropic MLV and the biological effect of the polypeptide envelope described by the present inventors, mediation of infection through a specific receptor, is generated solely by the amino acid sequence of said envelope. As known to a person skilled in the art, a codon of an amino acid can be generated by various nucleic acid sequences, due to the degeneracy of the genetic code thus the present invention relates to all isolated nucleic

acid sequences capable of encoding the envelope polypeptide sequences within the scope of the present invention

In a presently preferred embodiment, said nucleic acid sequence is capable of encoding an amino acid sequence that is at least 94% identical to the amino acid sequence as shown in
 5 SEQ ID NO 2, such as 94 5% identical, 95% identical, 95 5% identical, 96% identical, 96 5% identical, 97% identical, 97 5% identical, 98% identical, 98 5% identical, 99% identical, or 99 5 % identical

In a presently most preferred embodiment of the present invention, the isolated nucleic acid sequence of SL3-2 is as shown in SEQ ID NO 1

10 The mutated SL3-2 envelope polypeptide

In a sequence alignment between SL3-2 and MCF-247, three regions display differences in the amino acid sequence, as described in example 3 and figure 1 and 2 Two of these regions correspond to parts of the variable VRA and VRB regions, whereas the third is a 15 amino acids long stretch upstream of the proline rich region This region has been named
 15 VR3 by the present inventors

VR3 region

Further, a sequence alignment of MLVs from different sub-families show conserved amino acids at positions 203-208 WGLRLY and at positions 214-215 DP based on SL3-2 sequence, thus defining a 13 amino acid stretch of SEQ ID NO 2

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In the present context, the term "VR3 region" comprises all of the amino acids found between the residue found at two positions after the conserved tryptophan 197 and the residue before the conserved aspartic acid 214 (according to the sequence shown in SEQ ID NO 2) including these two positions

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The SL3-2 wild type envelope described in the present application contains an arginine residue in the amino acid position 212, see SEQ ID NO 2 The present application demonstrates that this amino acid is a most crucial amino acid which appears to be responsible for the limited tropism of the wild type SL3-2 envelope

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To further investigate the determinants of the differences in tropism between SL3-2 and MCF-247, chimeras were created in which the 15 amino acid VR3 region in SL3-2 was replaced by corresponding part of MCF-247, by use of an overlap extension method By replacing the VR3 region, the SL3-2 envelope was now capable of infecting a human cell by
 35 use of the human polytropic/xenotropic receptor encoded by the RMC1 locus for entry This shows that the tropism lies within the VR3 region of the SL3-2 virus

Further analysis showed that the merely a replacement of the arginine in position 212 of the VR3 region with a glycine could mediate infection of a human cell by the human
 40 polytropic/xenotropic receptor encoded by the RMC1 locus Glycine is the wild type amino acid found at the corresponding position in all other MLV strains Thus, the present

invention relates to a VR3 region with an arginine within said region at the corresponding position 212, but also relates to the replacement of said arginine

"Corresponding position" in the present context relates to two amino acids before the
5 conserved aspartic acid at position 214 in SL3-2 sequence

One embodiment of the present invention relates to a purified envelope polypeptide wherein said polypeptide includes at least one substitution in the VR3 region. In a presently preferred embodiment, the present invention relates to a purified envelope
10 polypeptide, wherein said mutation is at position 212 in SEQ ID NO 2

Glycine is the smallest amino acid with no significant side chain. It is possible that replacing this residue with an arginine, which has a much larger side chain, confers steric problems for binding of the SL3-2 envelope to the human polytropic receptor. If so,
15 replacing the glycine with other large amino acids is expected to result in changes in tropism. Examples of other substitutions which are likely to provide the same effect are alanine, asparagine, aspartic acid, cysteine, glutamic acid, phenylalanine, glycine, histidine, isoleucine, lysine, leucine, methionine, proline, glutamine, serine, threonine, valine, tryptophan or tyrosine.

20 Arginine is a basic amino acid and is positively charged in the physiological pH. The positively charged arginine in the wild type SL3-2 could enhance binding to the negatively charged glutamic acid in NIH 3T3 murine receptor while interfering with the positive charge of lysine found in receptors of other species.

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In a most preferred embodiment the mutation at position 212 in SL3-2 changes the arginine to glycine.

SL3-2 is the only MLV with an arginine at position 212. The conservation of glycine at this position in different sub-families of MLV seems to point toward a receptor independent
30 function for this region of envelope protein. The entry mechanism of MLV envelopes is largely unknown.

The present application describes a new surprising effect by a single mutation in the VR3 region of SL3-2 envelope polypeptides, thus a person skilled in the art would be able to
35 determine if further mutations could further alter and/or enhance the entry properties of these envelope polypeptides. Thus, another embodiment of the present invention relates to a purified envelope polypeptide wherein said polypeptide includes at least one substitution in the VR3 region, such as 1, 2, 3, 4, 5 or 6 substitutions in the VR3 region, see figure 1 and 2.

40 The present invention further relates to a purified mutated envelope polypeptide, wherein said at least one substitution alters the host tropism of a virus or an infectious particle displaying said polypeptide. A presently preferred embodiment of the present invention

relates to a, purified envelope polypeptide, wherein said purified polypeptide is a retroviral envelope polypeptide originally of murine origin capable of mediating infection of a human cell with a higher titer than a xenotropic or polytropic virus in a comparable system

- In the present context, an infectious particle is a particle comprising of various viral components that are assembled *in vitro* to a particle capable of mediating transduction (attachment fusion, reverse transcription and integration) of a retroviral vector One embodiment of the present invention relates to a mutated SL3-2 purified envelope polypeptide capable of mediating a higher infectivity in human cells than other MLV's, such as, but not limited to MCF-247, MCF-13 and X-MLV (NZB) viruses
- 10 In the present context, the term " higher infectivity" relates to any situation wherein the infection titre of one envelope is at least 2 fold higher than the other envelope with which it is compared

Further modifications to envelope

- Envelope proteins bind to cellular receptors and mediate infection by fusing the viral and cellular membranes Retroviruses, even very similar ones, utilise different cell surface receptors Receptor binding has been mapped to the N-terminal domain of SU subunit of envelope protein Accordingly, the largest differences in amino acid sequence among MLV sub-families are found in the N-terminal receptor-binding domain The receptors used by many retroviruses are house-keeping proteins such as amino acid transporters Therefore these viruses are not tissue specific and vectors using such envelopes will be able to infect the majority of the cells in an organism.

- In many of the predicted therapeutic applications of retroviral vectors, targeting specific cell types/tissues is vital and therefore changing receptor usage of envelope proteins is one of the major goals of the retroviral envelope research The fact that related envelope proteins can utilise different receptors has fuelled the idea that the tropism of envelopes can be changed by modifying the receptor binding domain by e g inserting non-viral sequences This modification could be in form of inserting peptide ligands or single chain antibodies against specific cell surface molecules Post-translational chemical modification of envelope proteins by attaching ligand molecules to envelope proteins might achieve the same goal Targeting specific cell types is possible in this way

- Several functional chimeric envelopes have already been described but none of these can mediate transduction at efficiencies comparable to the efficiencies obtained with wild type envelope proteins The described functional chimeric MLV-envelopes can be divided into two groups The first group has the heterologous ligand inserted in the N-terminal of the SU-protein and can mediate transduction without co-expression of wild type envelope, whereas the other group has the ligand inserted internally in SU and is dependent of co-expressed wild type envelope Peptide linkers and a single chain antibody specific for the human major histocompatibility complex class I (MHC-I) molecule have e g been inserted at four internal positions in Akv-env

The first attempts to direct virus particles towards receptors not normally recognised by retroviruses were done by antibody-bridging and by usage of chemical modifications. By cross-linking monoclonal antibodies against SU and the transferring receptor with a sheep anti-mouse kappa light chain antibody binding of the virus to human HEP2 cells, and subsequent internalisation was shown. However, internalisation of the virus by this infection route was not followed by establishment of the proviral state.

Others, used a similar approach to target the attachment of ecotropic viruses by streptavidin bridging biotinylated antibodies against SU and against specific membrane markers expressed on human cells. By this method human cells expressing MHC class I, MHC class II, epidermal growth factor and insulin were successfully infected, whereas this method did not prove feasible for promoting infection of cells expressing transferrin, high density lipoprotein and galactose receptors.

Also, chemically coupled galactose residues to ecotropic Env, making the virus particles capable of infecting human hepatoma cells through the asialoglycoprotein receptor, have been tried.

Infection of human cells by an ecotropic virus displaying chimeric-envelope proteins on the surface of the virion is also known to a person skilled in the art. This can be achieved by e.g. substituting a part of MoMLV SU with a sequence encoding the erythropoietin hormone (EPO), insertion of a sequence encoding human heregulin for infection of human breast cancer cells overexpressing the human epidermal growth factor receptor, substitution of an internal fragment of SU with a single-chain variable fragment (ScFv) derived from a monoclonal antibody recognising the human low density lipoprotein receptor which gave a chimeric envelope capable of infecting human cells.

In these reports with chimeric envelopes, targeted infection was only obtained when wild type env was co-expressed with the chimeric construct (from the Ψ 2 packaging cell line). This indicates that functional domains are contained within the ecotropic envelope, which is necessary for mediating infection beyond the point of receptor binding.

Various potential ligands have been used e.g. a ScFv against a specific hapten between amino acid 6 and 7 (position +7) in MoMLV SU, the amphotropic-MLV binding domain (208 amino acids), the polypeptide able to bind epidermal growth factor receptor (EGFR) (53 amino acids of EGF), collagen-binding properties, and a ligand (70 amino acids) recognising ErbB-3 and ErbB-4. The obtained targeting efficiencies with chimeric envelopes reported until now are considerably lower than the efficiencies obtained with wild type envelopes. The reasons for these low transduction efficiencies of target cells are probably diverse, including the choice of insertion site, stability of the chimeric envelope protein, the tertiary protein structure and the choice of target cells. Furthermore, the choice of ligand is probably also very important for obtaining infection, as several chimeric envelopes have failed to promote infection. One more positive example relates to insertion of a short

nondisruptive peptide (RDG) known to bind to several integrins displayed on the surface of cells (Golan TJ and Green-MR, 2002)

5 The above-described examples all utilised the ecotropic envelope. One advantage of using this envelope is that it is restricted in infecting human cells as the surface protein part of the envelope does not recognise a human receptor. The concept is that if the envelope can be engineered to bind to a human receptor by inserting a heterologous sequence in the envelope mediating this binding, the otherwise intact fusogenic properties of the envelope would mediate the fusion. Like the ecotropic envelope the SL3-2 envelope cannot mediate
10 infection of human cells. But by mutating a single amino acid this tropism can be changed making the SL3-2 envelope a much better scaffold than the ecotropic MLV envelope for genetic engineering of chimeric envelopes with a redirected specificity for a new cognate receptor.

15 Thus, a presently preferred embodiment of the present invention relates to a purified envelope polypeptide further comprising an inserted non-viral sequence, capable of redirecting the target cell specificity, by the resultant chimeric envelope.

Once the inserted non-viral sequence is placed within the envelope, a person skilled in the art would be capable of mutating essential amino acids of within said sequences similar to
20 e.g. the VR3 region. Thus the present invention a purified envelope polypeptide comprising an inserted non-viral sequence, wherein the chimeric envelope further contains secondary mutations, enabling activation of the fusogenic properties of said chimeric envelope, by binding of the receptor target. In a presently preferred embodiment of the present
25 invention said inserted sequence is a single chain antibody or e.g. a peptide ligand. Another embodiment of the present invention relates to a purified envelope according to the present invention further comprising a chemical modification of said envelope. In a presently preferred embodiment said chemical modification enhance and/or alters the host tropism.

30 One embodiment relates to a recombinant mammalian cell capable of displaying an envelope polypeptide according to the present invention.

The biological effect of any of the polypeptide envelopes described by the present inventors, is mediation of infection through a receptor. This mediation is generated solely by the polypeptide having the amino acid sequence of said envelope polypeptide. As
35 known to a person skilled in the art, a codon of an amino acid can be generated various nucleic acid sequences, thus the present invention relates to all isolated nucleic acid sequences capable of encoding an envelope polypeptide having an amino acid sequence as described in the present application. Thus, the present invention relates to an isolated nucleic acid sequence encoding any of the envelope polypeptides described in the present
40 invention.

In a presently preferred embodiment said isolated nucleic acid sequence is the nucleic acid sequence shown in SEQ ID NO 1 comprising the mutation at position 212, compared to the SL3-2 envelope polypeptide

Vectors and packaging cells

- 5 A recombinant mammalian expression vector in the present context comprises all vectors capable of directing expression of any given envelope by directing expression of vector DNA into RNA, poly-adenylation of said RNA, splicing of said RNA, if necessary, , export out of the nucleus of said RNA, and finally translation of said RNA outside of the nucleus
- 10 A replication competent retrovirus further comprises, all genes necessary for replication of a retrovirus, and for RNA being exported out of the cell and packaged in proteins expressed by said proteins Said RNA further comprises all RNA and DNA elements necessary for said RNA to be reverse transcribed into double stranded DNA and integrated into the host genome, as exemplified in figure 3 panel A In panel B this replication
- 15 competent retrovirus further comprises an ScFv or any heterologous peptide inserted into the envelope gene for redirection of host cell tropism Only the ScFv is depicted in figure 3, but other insertions such as an RGD peptide could be similarly useful

- The exemplified replication competent retroviral vector further comprises a replication
- 20 competent virus where a heterologous gene is being expressed from a position in the U3 region of the virus, panel C and D, or from a position in the 3 prime untranslated region downstream of the envelope and upstream of the downstream LTR, panel E and F Said replication competent vectors can further be redirected in host cell tropism by insertion of an ScFv or any heterologous peptide in the envelopes, panel D and F Only the ScFv is
- 25 depicted in figure 3 Based upon this example, the text of the present application and common knowledge of a person of ordinary skill in the art will be able to make other useful embodiments

- The term "a retroviral expression vector" comprises a retroviral vector being capable of
- 30 transcribed into RNA and capable of being packaged into a retroviral particle, reverse transcribed into double stranded DNA and inserted into the host genome by the retroviral enzymatic machinery For translation of said envelope an internal ribosome entry site (IRES) has been inserted upstream of the envelope in the exemplified retroviral expression vector, panel G and H The host cell tropism of said retrovirus can further be redirected by
- 35 inserting an ScFv or any heterologous peptide in the envelope, panel H Only the ScFv is depicted in figure 3, but other inserts could be similar useful

- One embodiment of the present invention relates to a replication competent retrovirus or any of the vectors described in the present application, comprising a purified retroviral
- 40 envelope polypeptide, capable of mediating infection of a cell, by use of the polytropic/xenotropic receptor encoded by the Rmc1 locus isolated from a NIH Swiss

inbred NFS/N mouse for entry, and unable of mediating infecting of a cell by use of a human polytropic/xenotropic receptor encoded by the RMC1 locus

Another presently preferred embodiment relates to a replication competent retrovirus or any of the vectors described in the present application, comprising a purified retroviral envelope polypeptide, capable of mediating infecting of a cell derived from *Mus musculus*,
 5 by use of the polytropic/xenotropic receptor encoded by the Rmc1 locus isolated from a NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of a human cell comprising a human polytropic/xenotropic receptor encoded by the RMC1 locus

Further, the present invention also relates to a replication competent retrovirus or any of
 10 the vectors described in the present application, comprising a purified murine retroviral envelope polypeptide, capable of mediating infection of a cell derived from *Mus musculus*, by use of the polytropic/xenotropic receptor encoded by the Rmc1 locus isolated from a NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of a human cell comprising a human polytropic/xenotropic receptor encoded by the RMC1 locus

15 In a presently preferred embodiment the present invention relates to a replication competent retrovirus or any of the vectors described in the present invention, comprising a purified envelope polypeptide having an amino acid sequence that is at least 94 % identical to an amino acid sequence such as shown in SEQ ID NO 2 94 %, or a fragment of said amino acid sequence that is at least 94 % identical to a fragment of an amino acid
 20 sequence such as shown in SEQ ID NO 2 94 %

Another presently preferred embodiment of the present invention relates to a replication competent retrovirus or any of the vectors described in the present application, comprising a purified envelope polypeptide, wherein said polypeptide includes at least one substitution in the VR3 region In a presently preferred embodiment, said mutation in the VR3 region is
 25 at position 212, as shown in SEQ ID NO 2 In a presently most preferred embodiment of the present application said mutation alters the host tropism of a virus or an infectious particle displaying said polypeptide

A further presently preferred embodiment of the present invention relates to a replication competent retrovirus or any of the vectors described in the present application, comprising
 30 a purified envelope polypeptide, wherein said purified polypeptide is a murine retroviral envelope polypeptide capable of mediating infection of a human cell

It is an object of the present invention to provide a replication competent retrovirus or any of the vectors described in the present application, comprising a mutated purified SL3-2 envelope polypeptide which is capable of mediating a higher infectivity in human cells than
 35 MCF-247, MCF-13 and X-MLV (NZB) viruses

Another object of the present invention is to provide a replication competent retrovirus or any of the vectors described in the present application, comprising any of the purified retroviral envelopes mentioned in the present application and further comprising an inserted non-viral sequence capable of redirecting the target cell specificity, by the

resultant chimeric envelope Another object of the present invention is to provide a replication competent retrovirus or any of the vectors described in the present application comprising a chimeric envelope, wherein said chimeric envelope further contains secondary mutations, enabling activation of the fusogenic properties of said chimeric

5 envelope, by binding to the receptor target

A presently preferred object of the present invention is to provide a replication competent retrovirus or any of the vectors described in the present application, comprising any of the purified retroviral envelopes mentioned in the present application and further comprising an inserted non-viral sequence, wherein said inserted sequence is a single chain antibody

10 Another object of the present invention is to provide a replication competent retrovirus or any of the vectors described in the present application comprising any of the purified retroviral envelopes mentioned in the present application and further comprising a chemical modification of said envelope

A preferred object of the present invention is to provide a replication competent retrovirus
15 or any of the vectors described in the present application, comprising any of the purified retroviral envelopes mentioned in the present application and further comprising a chemical modification of said envelope, wherein said chemical modification enhances and/or alters the host tropism

A particular embodiment of the present invention relates to any of the replication
20 competent vectors described in the present application and further comprising a heterologous translation cassette

A presently preferred particular embodiment relates to a replication competent vector comprising a heterologous translation cassette, wherein said heterologous translation cassette comprises an IRES-gene element

25 Another particular embodiment of the present invention relates to a vector according to the present invention further comprising at least one heterologous gene to be expressed

In a presently preferred particular embodiment, the present application relates to a vector according to the present invention further comprising at least one heterologous gene to be expressed, wherein said expression is directed by a IRES-element

30 Retroviral genomes can be divided into two different functional parts The protein coding genes are not necessary as *cis* elements (RNA or DNA in the present context) sequences for viral replication cycle Only their encoded proteins have a role to play In contrast there are a few other elements in a retroviral genome that are necessary for viral replication The packaging signal is one such element and is necessary for packaging of the viral
35 genome into budding virions

A retroviral vector technology is based on separation of these *cis* and *trans* elements and has two major elements a vector and a packaging cell line A simple vector is a viral

genome in which the protein-coding genes are replaced by a heterologous sequence. The packaging cell line is engineered to produce the viral Gag, Pol and Env proteins from constructs that lack the packaging signal (to prevent them from being taken up by budding virions). Thus, when a vector is inserted into a packaging cell line, it will be packaged into budding virions and can be transferred into target cells.

MLV based packaging cells are widespread tools for research. Packaging cells based on ecotropic viruses have the advantage of being harmless to humans and are used in bio-safety level 1 laboratories. Since all ecotropic envelopes use the same receptor, superinfection resistance might necessitate the use of virions other than ecotropic, if infection of an ecotropic based packaging cell line is desired. So far, the choices in these situations have been an amphotropic based cell line despite the greater safety concerns involved.

The term "superinfection resistance" is used in the present context to describe the fact that viral infected cells express the viral envelope protein on their surface. Here the envelope protein and the cellular receptor interact with one another and receptors are thereby masked from external viral particles. Thereby an infected cell cannot be infected again by the same virus genus or by any other virus that use the same receptor for entry. This phenomenon is also called interference and is a convenient means of determining if two different viruses utilise the same receptor.

A packaging cell line based upon the envelope polypeptides of the present invention can replace the amphotropic cell lines and in the same time, have the benefit of higher safety. A packaging cell line using the envelope polypeptides of the present invention may also be more effective in infecting murine cell types with low expression of the ecotropic receptor. One embodiment of the present invention relates to a packaging cell construct comprising a recombinant mammalian expression vector comprising a nucleic acid coding for a polypeptide envelope as described in the present application, and a non-viral or viral promoter and poly-adenylation signals.

Another embodiment of the present invention relates to use of any of the vectors according to the present invention for the generation of a packaging cell.

The mini-virus system described below is a variation of the retroviral vector technology. The main difference is that in the mini-virus system the envelope protein is expressed from the vector instead of the cell line. The complementing cell line for this vector contains only the viral *gag* and *pol* genes and is referred to as a semi-packaging cell line.

One embodiment of the present invention relates to use of a vector (mini-virus) in a cell constitutively, or inducible, or in any other way expressing the *gag/pol* genes of simple retroviruses.

Gene Therapy

In vivo gene therapy also necessitates avoiding immune responses towards gene delivery vectors. Wild type envelope proteins are antigenic and modifications of their epitopes might help to avoid the immune system. For gene therapy purposes, especially human gene therapy, an envelope protein that is unable to infect human cells, such as that of SL3-2, is preferable as a platform for creating modified envelopes, since secondary unspecific infections are unlikely to occur. Attempts to redirect ecotropic envelope have not been very successful. The present invention shows that the SL3-2 can be mutated to infect human cells. Therefore, the SL3-2 envelope might prove to be a better platform for targeting specific human cells.

Thus, the present invention relates to use of a packaging cell as described above, for the preparation of a medicament for the modification of cells by genetic engineering or to provide an active substance in the context of gene therapy.

Further, the present invention also relates to use of any of the vectors described above, for the preparation of a composition for the modification of a cells by genetic engineering or to provide an active substance in the context of gene therapy.

Disease models

By including into a replication competent virus an additional gene, an oncogene, a proto-oncogene or a dominant-negative form of a tumor-suppressor gene, new MLV-based models for multistep oncogenesis have been established. This strategy aim at analysing co-operating events resulting from the introduced gene and the effect on host genes caused by proviral insertion. The first integration of a provirus harbouring a gene with oncogenic activity will provide two hits, one resulting from the introduced gene and one from the effect at the site of integration. Such models resemble transgenic and knockout mouse models where one activating event is introduced in the germ-line. However, the results may differ because the gene with oncogenic activity will not be present in the embryo and will only enter cells together with the virus.

In general, the murine leukaemia viruses (MLVs) constitute a polymorphic group of non-acute transforming retroviruses. The pattern of disease induction varies among individual strains and mutants and also depends upon the specific laboratory mouse strain.

Although the overall pathogenesis of MLV infections is still poorly understood, insertional mutagenesis of critical host genes by proviral integration has been established to be of importance for induction of lymphomas and leukaemia's. Differences in long terminal repeat (LTR) enhancer sequences associated with binding of specific host transcription factors contribute to variation in the pathogenic potency and specificity among MLV variants and mutants. However, differences in other parts of the genome can also be of importance e.g. the envelope. Retroviral models have the advantage that critical host genes can be identified through tagging by integrated proviruses or by means of the interaction of their products with parts of the viral machinery. Rapid advances in genome

analysis shorten the path to critical host genes of the animal model and to their human counterparts

Thus, it is an object of preferred embodiments of the present invention to provide
 5 retroviruses expressing a retroviral envelope polypeptide of the invention as these viruses may infect a different population of target cells than do the ecotropic envelopes. By targeting a different subset of cells these viruses may invoke different sets of critical host genes and thereby further exploit the information from proviral integration sites in available tumours towards the identification and functional characterisation of critical host
 10 genes involved in oncogenesis

The advantages of using a retroviral envelope polypeptide having the properties of a wild type SL3-2 envelope as compared to MCF envelopes are that these envelopes do not mediate infection of human cells. By displaying such an ecotropic trait, animal experiments
 15 involving viruses expressing a phenotype like the wild type SL3-2 envelope, can be performed under the lowest classification for genetically engineered cells or animals facility e.g. bio-safety level 1 according to Danish safety regulations

One embodiment of the present invention relates to the use of a virus or vector according
 20 to present invention for gene discovery by infection of a new-born rodent

In a presently preferred embodiment of the present invention, said rodent constitutively express the gag/pol genes of simple retroviruses, or said rodent express the gag/pol genes of simple retroviruses in a tissue specific manner, or the expression of the gag/pol genes of simple retroviruses is developmentally regulated said rodent, or said rodent express the
 25 gag/pol genes of gamma retroviruses both tissue specifically and in a developmentally regulated manner

A particular embodiment of the present invention relates to a method for gene discovery comprising

- a) using a virus or vector according to the present invention
- 30 b) infecting a new-born rodent with said virus or vector
- c) inducing a tumour by means of said virus or vector
- d) isolating said tumour in said rodent e) identifying a gene involved in the oncogenesis by cloning the integration site of said virus or vector in said tumour

In a presently most preferred embodiment, the present invention relates to a method for
 35 gene discovery of cancer related genes

Safety

The degree to which laboratory security is implemented should be commensurate with risk. All laboratories, including those handling only low-risk biological materials under bio-safety level 1 containment practices, must maintain a basic level of security.

- 5 There are several bio-safety concerns that arise with the use of viral vectors including

Tropism (host range) – viral vectors that can enter (infect) human cells are often used

Replication-deficient viral vectors can gain back the deleted genes required for replication (become replication-competent) through recombination - referred to as replication-

- 10 competent virus (RCV) breakouts

Genes may be expressed in tissues and/or organisms where they are normally not expressed. In the case of some genes such as oncogenes this could have far-reaching negative consequences.

- 15 When evaluating safety for use of viral vectors, there are a number of factors that need to be considered including risk group of the organism, tropism (organism and tissue), route of transmission, whether the virus integrates into the host genome, and the specific gene(s) being introduced.

- 20 Viral vectors frequently used are retrovirus/lentivirus, adenovirus, adeno-associated virus, poxvirus, herpes virus, and baculovirus. Amphotropic Moloney murine leukaemia virus (MMLV) and adenovirus are common viral vectors used to introduce genes into human cells.

- 25 NIH classifies amphotropic MMLV as RG2 pathogens, and thus MMLV viral vectors infect human cells and have potential for RCV breakthroughs. Amphotropic MMLV integrates into the host's genome and translates into stable expression of introduced genes and the potential for insertional mutagenesis of host genes.

- 30 There are a number of ways to improve safety when working with viral vectors e.g. to consider the alternative of nonviral vectors, limit tropism or use ecotropic MMLV with methods that allow the virus to enter human cells in a limited manner. This strategy is especially relevant for introduction of genes such as oncogenes, mutant tumor suppressor genes, mutant repair genes, and some signal transduction pathway genes.

- 35 Thus, a particular embodiment of the present invention relates to the use of any of the envelope polypeptides or vectors thereof having the properties of wild type SL3-2 in a bio-safety level 1/PS I/PSI laboratory animal facilities or equivalents thereof.

- 40 By co-cultivation of two packaging cell lines each using a different receptor, it is possible to ping-pong the transducing vector back and forth between the two populations of packaging cells. As there will be no restriction to infection from a vector packaged in an envelope of the other packaging cell line this ping-pong effect of the vector will increase the number of vectors in the cells, and thereby increase the expression level of said vector.

By using a packaging cell line having the properties of wild type SL3-2 in conjunction with an ecotropic receptor, no virus particle will be made capable of infecting human cells and the experiment can thus be performed under the lowest level of classification regarding
5 genetic modified cells or animals (e g class 1 according to Danish safety regulations)

SEQ ID's

SEQ ID NO 1 (nucleic acid sequence of SL3-2)

ATGGAAGGTCCAGCGTTCTCAAAACCCCTTAAAGATAAGATTAACCCGTGGGGCCCCCTAATAGTC
 5 CTGGGAATCTTAATGAGGGCAAGAGTATCAGTACAACATGACAGCCCTCATCAGGTCTTCAATGTT
 ACTTGGAGAGTTACCAACTTAATGACAGGACAAACAGCTAATGCTACCTCCCTCCTGGGGACAATG
 ACCGATGCCTTTCCTAAACTGTACTTTGACTTGTGCGATTTAATAGGGGACGACTGGGATGAGACT
 GGACTCGGGTGTGCGACTCCCGGGGGAAGAAAAAGGGCAAGAATATTTGACTTCTATGTTTGCCCC
 GGTCACACTGTGCTAGCAGGGTGTGGAGGGCCGAGAGAGGGCTACTGTGGCAAATGGGGATGTG
 10 AGACCACTGGACAGGCATACTGGAAGCCATCATCATGAGGACCTAATTTCCCTTAAGCGAGGAA
 ACACTCCTAAAGGCCAGGGCCCCCTGTTATGATTCTCGGTGGTCTCCAGTAGCGCCAGGGTGCCA
 CACCGGGGGGTGATGCAACCCCTAGTCTAGAAATCACTGACGCGGGTAAAGGGGCCAGGCTGG
 GACGCCTCAAAGCATGGGGACTAAGACTGTACCGATCCACAAGGACCGACCCGGTGACCCGGTT
 CTCTTTGACCCGCCAGGTCTCAATATAGGGCCCCGCGTCCCCATTGGGCCTAATCCCGTGATCAT
 15 TGACCAGTTACCCCCCTCCCGACCCGTGCAGATCATGCTCCCCAGGCCTCCTCAGCCTCCTCCACC
 AGGCGCAGCCTCTACAGTCCCTGAGACTGCCCCACCTTCCCAACAACCTGGGACGGGAGACAGGC
 TGCTAAACCTGGTAAATGGAGCCTACCAAGCTCTCAACCTCACCAGTCTGACAAAACCCAAGAGT
 GCTGGTTGTGTCTGGTAGCGGGACCCCCCTACTACGAAGGGGTTGCCGTCTAGGTACTTATTCCA
 ACCATACCTCTGCCCCAGCTAACTGCTCCGTGGCCTCCCAACACAAGCTGACCCTGTCCGAAGTGA
 20 CCGGACAGGGACTCTGCGTAGGAGCAGTTCCCAAACCCATCAGGCCCTGTGTAATACCACCCAG
 AAGACGAGCAACGGGTCCTACTATCTGGCTGCTCCCGCCGGGACCATTGGGGCTTGCAACACCGG
 GCTCACTCCCTGCCTATCTACCACTGTGCTCGACCTCACCACCGATTACTGTGTCCTGGTTGAGCTC
 TGGCCAAAAGTGACCTACCACTCCCCTGGTTATGTTTATGGCCAGTTTGAAGAAAAAACCAATATA
 AAAGAGAACCCGTCTCACTAACTCTGGCCCTACTATTAGGAGGACTCACTATGGGCGGAATTGCCG
 25 CCGGAGTGAGGAAACAGGGACTACCGCCCTAGTGGCCACTCAGCAGTTCCAACAACCTCCAGGCTGCC
 ATGCAGGATGACCTTAAAGAAGTTGAAAAGTCCATCACTAATCTAGAAAGATCTTTGACCTCCTTGT
 CCGAAGTAGTGTTACAGAATCGTAGAGGCCTAGATCTACTATTCTTAAAGAGGGAGGTTTGTGTG
 CTGCCTTAAAGAAGAATGCTGTTTCTATGCCGACCACACAGGATTGGTACGGGATAGCATGGCCA
 AACTTAGAGAAAGATTGAGTCAGAGACAAAACCTTTGAATCCCAACAAGGGTGGTTTGAAGGGC
 30 GTTTTAAACAAGTCCCCTTGGTTCACCACCCTGATATCCACCATCATGGGTCCCCTGATAATCCTCTT
 GTTAATTTTACTCTTTGGGCCTTGATTCTCAATCACCTGGTCCAGTTTATCAAAGACAGGGTTTCG
 GTAGTGCAGGCCCTGGTCCTGACTCAACAATATCATCAACTTAAGACAATAGAAGATTGTGAATCAC
 GTGAATAA

35 SEQ ID NO 2 (amino acid sequence of SL3-2)

MEGPAFSKPLKDKINPWGPLIVLGILMRARVSVQHDSPHQVFNVTVRVTNLMTGQTANATSLTGTM
 DAFPKLYFDLCLIGDDWDETLGCRTPGGRRKRARIFDFYVCPGHTVLAGCGGPREGYCGKWGCETT
 GQAYWKPSSSWDLISLKRGNTPKGQGPCYDSSVSSSAQGATPGGRCNPLVLEFTDAGKRASWDAS
 40 KAWGLRLYRSTRDPVTRFSLTRQVLNIGPRVPIGNPNVIIDQLPPSRPVQIMLPRPPQPPPPGAASTVP
 ETAPPSQQPGTGDRLLNLVNGAYQALNLTSPDKTQECWLCLVAGPPYYEGVAVLGTYSNHTSAPANCS
 VASQHKLTLEVTGQGLCVGAVPKTHQALCNTTQKTSNGSYLLAAPAGTIWACNTGLTPCLSTTVLDL
 TTDYCVLVELWPKVITYHSPGYVYGQFEEKTKYKREPVSLTLALLGGLTMGGIAAGVGTGTTALVATQQ

FQQLQAAMQDDLKEVEKSITNLERSLTSLSEVVLQNRRLDLLFLKEGGLCAALKEECCFYADHTGLVR
DSMAKLRERLSQRQKLFESQQGWFEGLFNKSPWFTTLISTIMGPLIILLILLFGPCILNHLVQFIKDRVS
VVQALVLTQQYHQLKTIEDCESRE

Legends to figures

Figure 1

Alignment of amino acid sequences of selected MLVs in the VR3 region. Residues corresponding to arginine 212 are shown in the rectangular box

5

Figure 2

Amino acid aligning of SL3-2 and MCF 247 envelopes. The non-homologous regions of SU, including VR3 region, are marked by brackets

Figure 3

Depicted in panel A is a wild type replication competent virus

In panel B said replication competent virus has an ScFv insert in the envelope for redirection of tropism

15

Panel C and E are replication competent vectors where an heterologous translational cassette has been inserted into either the U3 region panel C or in the 3' un-translated region downstream of the envelope gene Panel E

20 Panel D and F are the same as panel C and E except a ScFv has been inserted in the envelope for redirection of tropism

Panel G is a replication competent retroviral vector expressing an envelope gene from a mono-cistronic mRNA by directing translation of the envelope gene by an internal ribosome entry site (IRES)

25

Panel H same as Panel G except that a ScFv has been inserted into the envelope gene for redirection of tropism. In the present context ScFv means a Single chain antibody or any other heterologous peptide sequence that mediates redirection of envelope tropism

30

Figure 4

Library analysis of SU. An overlap extension reaction was used to create the DNA library, which is subsequently transfected into Plat E packaging cells. The packaging cells contain wild type envelope protein and therefore produce infectious particles independent of the mini-virus envelope genes. A stable population of infected semi-packaging cells (the cellular library) is established using these virions. The cells in the library produce virions that bear envelope mutants on the surface and contain the corresponding gene. Target cells are transduced and selected. The surviving colonies contained mini-viruses that encode functional envelopes

40

Figure 5

Semi-packaging cells containing a mini-virus vector produce virions. The mini-virus bearing virions can infect both semi-packaging cells and other permissive cells, but only semi-packaging cells are able to produce new virions. Mini-virus is thus conditional replication competent.

Figure 6

The genomic structure of the mini-virus vector. All of the elements except *neo*, IRES and *env* are derived from Akv MLV.

Examples

Materials and methods

Cells

Murine fibroblast cell line NIH 3T3 and NIH 3T3 derived packaging cell line Psi-2 (Mann *et al*, 1983) and semi-packaging cell line CeB (Cosset *et al*, 1995) were grown in Dulbecco's modified Eagle's medium with glutamax-1 (Gibco BRL, Life Technologies) containing 10% (vol/vol) newborn calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The CeB semi-packaging cell line was selected with the same growth medium containing 40 µg/mL blasticidin.

Human HeLa and TE 671 cells were grown under the same conditions as murine NIH 3T3 cells. Human HeLa mCAT (Aagaard *et al*, 2002) cells were selected with the above described medium containing 75 µg/mL Hygromycin B, and no blasticidin.

BOSC 23 packaging cells that are derived from the Ad5 transformed human embryonic kidney 293T cell line (Pear *et al*, 1993) were selected with 250 µg/mL xanthine (Sigma), 25 µg/mL mycophenolic acid (Gibco BRL, Life Technologies), 20 µg/mL aminopterin (Sigma) and 60 µg/mL thymidine (Sigma) in Dulbecco's modified Eagle's medium containing 10% (vol/vol) dialyzed fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin.

The Plat E packaging cell line is also derived from human embryonic kidney 293T cell line (Monta *et al*, 2000) and was selected with 10 µg/mL blasticidin and 1 µg/mL puromycin in Dulbecco's modified Eagle's medium with glutamax-1 (Gibco BRL, Life Technologies) containing 10% (vol/vol) fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin. While not under selection, both Plat E and BOSC 23 cells were grown in Dulbecco's modified Eagle's medium with glutamax-1 (Gibco BRL, Life technologies) containing 10% (vol/vol) fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin.

All cells were incubated at 37°C in 90% relative humidity and 5% CO₂.

Transfections and transduction/titer measurements

Transfections were done by the calcium phosphate precipitation method without a glycerol shock (Graham *et al* , 1973) and leaving the calcium precipitate on the cells for 24 hours

- 5 Transfections of plasmids were done using 10 μ g of the plasmid in question and 1 μ g of an EGFP expressing plasmid (Clonetech) EGFP was included as a visual aid to determine the success of transfections

- 10 Transfection of PCR products was achieved by adding to the approximately 300ng of PCR product 10 μ g of plasmid pUC19 as carrier DNA and 1 μ g of EGFP expressing plasmid

- 15 Medium for the transfected cells was renewed every day until it was used for transduction, usually between 48 and 72 hours after transfection, depending on the number of green fluorescing cells The day before transduction, medium was changed from bovine fetal serum to newborn calf serum containing medium

- 20 Target cells were seeded at concentration of 5*10³ cells/cm² 24 hours before transduction Supernatant from transfected cells was filtered (0.22 μ m) The supernatant with 6 μ g/mL polybrene was added either by 10-fold end-point dilution (to NUNC six-well-dishes for measuring titers) or crude (for creating pool of infected semi-packaging cells) to target cells, depending on the experiment

- 25 24 hours later, the infected cells were selected using medium as described (DMEM with 10% NCS and 1% penicillin/streptavidin supplemented with 600 microgram G418 per ml) (Gibco BRL, Life Technologies,) before containing 600mg/mL G418 for 10-12 days

Overlap extension reactions

Downstream fragments

- 30 The Polymerase Chain Reactions (PCR) for synthesizing the overlap extension fragments were done using Expand High Fidelity PCR System (Roche) and 5ng template per 100 μ L reaction and 25 amplification rounds

100 μ L reaction contained 10 μ L 10 x buffer, 0.8mM dNTP (0.2mM of each nucleotide), 0.25pM of each primer and 2.625 units of enzyme, as above

- 35 94°C 2 min , 25 x (94°C 1 min, 50°C 1 min , 72°C 2 min 30 sec), 72°C 7 min

The primer (111019 AACAAATTTACACAGGAAACAGC) was used as the end primer in all reactions Other primers used are shown in table 1

Table 1 Downstream primers

Library	Primer name	Primer Sequence

SL3-2/MCF VRA	B8142D01	GTTTGCCCCGGTCACACTGTGCCAACAGGGTGTGGAGGGCCG AGA
SL3-2/MCF VRB	A0341A02	CTTAAGCGAGGAAACACTCCTCAGAATCAGGGCCCCCTGTTATG ATTCTCAGCGGTCTCCAGTGACATCAAGGGTGCCACACCGG GGGGTCGA
SL3-2/MCF VR3	A0341A01	GGTAAAAGGGCCAGCTGGGACGGCCCCAAAGTATGGGGACT AAGACTGTACCGATCCACAGGGATCGACCCGGTGACCCGGTT CTCT
SL3-2/MCF Leader	B8142D02	CTAATAGTCCTGGGAATCTTAATAAGGGCAGGAGTATCAGTAC AACATGACAGC
SL3-2/MCF alternating VR3 library	A0571A05	GGTAAAAGGGCCAGCTGGGACGSNYCNAAGYNTGGGGACT AAGACTGTACCGATCCACARGRAYHGACCCGGTGACCCGGTT CTCT
SL3-2/MCF VR3 RT	T3130F07	GGTAAAAGGGCCAGCTGGGACGCCTCCAAAGCATGGGGACTA AGACTGTACCGATCCACAAGGACCGACCCGGTGACCCGGTTC TCT
SL3-2/MCF VR3 RI	T3130F08	GGTAAAAGGGCCAGCTGGGACGCCTCCAAAGCATGGGGACTA AGACTGTACCGATCCACAAGGATCGACCCGGTGACCCGGTTC TCT
SL3-2/MCF VR3 GT	T3130F09	GGTAAAAGGGCCAGCTGGGACGCCTCCAAAGCATGGGGACTA AGACTGTACCGATCCACAGGGACCGACCCGGTGACCCGGTTC TCT
SL3-2/MCF VR3 GI	T3130F10	GGTAAAAGGGCCAGCTGGGACGCCTCCAAAGCATGGGGACTA AGACTGTACCGATCCACAGGGATCGACCCGGTGACCCGGTTC TCT

Upstream fragments

The upstream fragments were made using the primer (111020 GATTAAGTTGGGT AAGCCAGGG) as the end primer. Other primers used are shown in table 2. PCR conditions were otherwise the same as those for downstream fragments.

Table 2 Upstream primers

Construct	Primer name	
SL3-2/MCF VRA	B8087E02	CACAGTGTGACCGGGGCAAAC
SL3-2/MCF VRB	B8087E04	AGGGAGTGTTCCTCGCTTAAG
SL3-2/MCF VR3	B8087E03	GTCCCAGCTGGCCCTTTTACC
SL3-2/MCF Leader	B8087E05	TAAGATCCCAGGACTATTAG

The fragments were purified from 1% agarose gels (by GFX PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech).

The overlap extension reaction was done using rTth DNA Polymerase XL (PE Biosystems) in ten rounds using end primers (111019 and 111020) and equimolar amounts of the two fragments

- 5 100µL reaction contained 30µL 3.3 XL buffer II, 1.375 mM Mg(OAc)₂, 0.8mM dNTP (0.2mM of each nucleotide), 0.25pM of each primer and 4 units of enzyme, as above

95°C 3 min , 10 x (95°C 1 min , 60°C 30 sec , 73°C 6 min), 73°C 8 min

- 10 The resulting overlap extension product was purified by Wizard DNA Clean-Up System (Promega) and used for transfection

Example 1 Generating the envelope protein of SL3-2

- 15 The envelope protein of SL3-2 was taken from genomic DNA of NIH 3T3 cells infected with SL3-2 virus. PCR was used to amplify the envelope. The upstream primer was chosen to match a conserved sequence upstream of the splice acceptor site among different MLV strains. The downstream primer was designed according to the known sequence of SL3-2 LTR (Dai *et al* , 1990). The amplified PCR fragment was subsequently cloned into the mini-
- 20 virus to replace the original Akv envelope. The new construct was designated NeoSL3-2mo. Three clones were chosen for sequencing.

- One of the clones contained a frameshift mutation and was not infectious. This SL3-2 envelope shows a 92% homology on nucleotide level with the envelope protein of MCF-247
- 25 polytropic MLV. The latter has a wide host range and is able to infect cells from many species (Rein 1982) , (Hartley *et al* , 1977), (Chattopadhyay *et al* ,1982), whereas the original reports claimed that SL3-2 has the same host range as the ecotropic viruses (Pedersen *et al* , 1981), (Rein *et al* , 1984)

30 Cloning of SL3-2 envelope

The envelope of SL3-2 was amplified by the following PCR from the genomic DNA of infected NIH 3T3 cells

PCR conditions

35

10µL 10 x buffer, 0.8mM dNTP (0.2mM of each nucleotide), 0.25pM of each primer and 2.625 units of enzyme (Expand High Fidelity PCR System (Roche))

Using primers

- 40 204820 CTCTCCAAGCTCACTTACAGGCCCTC
205585 TGCGGCCGCGTCGACTGGCTAAGCCTTATGAA

95°C 2 min , 45 x (95°C 1 min, 60°C 30 sec , 73°C 4 min), 73°C 5 min

We have two different mini-virus vectors with Akv envelope that differ in the length of the spacer between the IRES element and *env* gene

- The Neo-env-mo vector contains a linker similar in size to the one found in wild type EMCV (Morgan et al 1992, Bachrach et al 2002), while Bi-neo-env contains a longer linker with multiple cloning sites similar to the linker found in pJD214HyBi+ (Koo et al 1992)

Spacer sequences in Bi-neo-env and Neo-env-mo are shown below The ATG start codon of Env is shown in *italics* The bold T (T) in the sequence corresponds to T827 in EMCV

- 10 nucleotide coordinates (Jang and Wimmer 1990)

Bi-neo-env AACACGATTGCCGCGTGCGGCCGCTAACACTCCGGAGCTCGAGCCAATATG
Neo-env-mo AACACGAT-----AATACCATG

- 15 The PCR fragment was purified from agarose gel (GFX PCR DNA and gelband gel purification kit from Amersham Pharmacia Biotech) and used to replace the fragment removed from pBiNeoEnv plasmid by NotI (Gibco BRL, Life Technologies) and CclII restriction enzymes (Amersham Lifescience) The new plasmid was named pBiNeoSL3-2 Subsequently, both pBiNeoSL3-2 and pNeoEnvmo were cut by NcoI (Gibco BRL, Life Technologies) The cut vector (pBiNeoSL3-2) was after dephosphorylation by shrimp alkaline phosphatase from Roche according to manufacture and ligated with the NcoI fragment of pNeoEnvmo resulting in the plasmid pNeoSL3-2mo

Example 2 Determining host range and receptor usage of the new mini-virus

- 25 To determine the host range of the cloned mini-virus, BOSC 23 packaging cells were transfected by two clones of NeoSL3-2mo 24 hours later supernatant from these were used to transduce semi-packaging cells (CeB cells) CeB cells were selected for G418 resistance until they were confluent The resistant semi-packaging cells contain the vector Supernatant from these were used to measure the titer of vectors bearing SL3-2 envelope on a number of different cells Murine NIH 3T3 and human HeLa cells were used Psi-2 cells are packaging cells that express ecotropic envelope constitutively and thus block infection by ecotropic viruses NIH 3T3 cells infected by MCF 247 block infection through polytropic receptor HeLa cells that express ecotropic receptor mCAT-1 were also included to ensure that mini-virus is capable of infecting human cells

35

Table 3 The tropism of SL3-2 mini-virus Titers measured in cfu/mL

Mini-virus bearing Env from	NIH3T3	PSI-2	NIH 3T3/ MCF 247	HeLa mCAT	HeLa
Akv clone #1	$1,6 \cdot 10^6$	$1,7 \cdot 10^2$	$4 \cdot 10^5$	$5 \cdot 10^4$	0
Akv clone #2	$8 \cdot 10^5$	$1,2 \cdot 10^2$	$2,6 \cdot 10^4$	$3 \cdot 10^4$	0
SL3-2 clone #1	$1,2 \cdot 10^5$	$1,7 \cdot 10^4$	<1	0	0

SL3-2 clone #2	8*10 ⁵	6*10 ⁵	2	0	<1
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As can be seen in table 3 the tropism of SL3-2 mini-virus vectors bearing the Akv or SL3-2 envelopes have similar titers on NIH 3T3 cells. Expression of ecotropic envelope in PSI-2 cells lowers the titer of ecotropic mini-virus by a factor of around 10 000, but have no effect on the titer of SL3-2 bearing mini-virus. NIH 3T3/MCF 247 shows interference with SL3-2 envelope but not with that of Akv. The reason for lower interference levels seen in Psi-2 cells compared with MCF 247 infected NIH 3T3 cells is most likely that Psi-2 cells express ecotropic envelope less efficiently. This is not surprising since Psi-2 cells do not contain a replication competent virus. These data prove that SL3-2 uses polytropic and not the ecotropic receptor (mCAT-1). None of the envelopes in this study were able to mediate infection of human HeLa cells and the fact that HeLa cells that express ecotropic receptor are infected with mini-virus bearing the ecotropic envelope indicates that in the case of SL3-2 mini virus, infection is prohibited because of lack of envelope/receptor interactions. These results were confirmed in repeated experiments.

15

Example 3 Determinants of the limited host range of SL3-2

Three regions showed amino acid differences between SL3-2 and MCF 247. Two of these regions correspond to parts of the variable VRA and VRB regions, whereas the third was a fifteen amino acids long stretch upstream of the proline rich region. To further investigate the determinants of the differences in tropism of these two viruses, chimeras were created in which these segments in SL3-2 were replaced by those of MCF 247, using the described overlap extension method (Jespersen et al , 1997).

Clone 1 of pNeoSL3-2mo was used in this study. As control, another chimera was created, in which the signal peptide of MCF 247 replaced that of SL3-2. There are two amino acid differences in the signal peptide, but since the signal peptide is not found in the mature envelope, no change of tropism was expected. The first three chimeras were named S/M Leader, S/M VRA and S/M VRB according to the segment that was changed. The construct in which the proline rich proximate region was changed was named S/M VR3.

30

These constructs were transfected into Plat E packaging (Morita et al , 2000). The produced virions were used to infect CeB semi-packaging cells. After selection of these for G418 resistance, titer of the mini-virus constructs were measured on NIH 3T3 murine cells, human TE 671 and HeLa cells, NIH 3T3 cells infected with MCF 247 and Psi-2 cells.

35

Table 4 Receptor usage of SL3-2/MCF 247 chimeras. Titers measured in cfu/mL. ND not determined

	NIH 3T3	NIH 3T3/ MCF 247	Psi-2	HeLa	TE 671
S/M Leader	2*10 ⁵	12,5	1*10 ⁵	0	5
S/M VRA	2,25*10 ⁵	2,5	4*10 ⁵	0	2,5

S/M VRB	3×10^6	2,5	5×10^5	0	5
S/M VR3	5×10^5	2,5	3×10^5	$2,5 \times 10^2$	$2,25 \times 10^4$
NeoSL3-2mo	3×10^5	0	1×10^5	0	ND

As can be seen in table 4, all of the constructs have comparable titers on NIH 3T3, MCF 247 infected NIH 3T3 and Psi-2 cells, indicating that all of the four chimeras utilize the same receptor with same efficiency in murine cells. Interestingly, the S/M VR3 construct had a 5,000 to 10,000 fold higher titer on human TE 671 cells than other constructs and was the only mini-virus capable of infecting human HeLa cells, indicating that a major determinant for utilizing the human receptor is to be found in this region.

Among the amino acids in the VR3 region, there are five that are different between SL3-2 and MCF 247. Any combination of these can be responsible for the different tropisms of these viruses.

To clarify this issue, a randomized library was created in which the amino acids found in the five positions alternated between those found in SL3-2 or MCF 247 by the described overlap extension method.

The product of the overlap extension reaction was transfected into Plat E packaging cells. These cells express Gag, Pol and Env proteins of Moloney MLV. Supernatant containing virions was harvested after 48 hours and used to transduce CeB semi-packaging cells. Infected cells were selected on the basis of their resistance to G418. This ensures that the populations of semi-packaging cells contain stably integrated vectors. CeB cells express moloney MLV Gag and Pol but not Env. Therefore any virions produced will carry the envelope protein encoded by the integrated vector. Since the same vector is packaged into the virion, the gene encoding the envelope protein will be integrated in the target cell, if the virion is infectious. Infected CeB cells were grown under G418 selection until they were confluent. Supernatant was harvested from these and used to transduce TE671 human cells seeded in petri-dishes (NUNC P10). Petri-dishes were chosen to facilitate isolation of colonies that would form. Colonies were isolated and the sequences of integrated vectors were determined. See figure 4. The library was designed so that each alternating amino acid could be translated from at least two different codons. This was done to distinguish any functional requirements of nucleotide sequence from those on the amino acid level. 34 colonies were isolated and sequenced.

The results are presented in table 5.

Table 5 Colonies isolated from alternating SL3-2/MCF 247 VR3 library

Randomised positions									
SL3-2	MCF 247	SL3-2	MCF 247	SL3-2	MCF 247	SL3-2	MCF 247	SL3-2	MCF 247
Ala	Gly	Ser	Pro	Ala	Val	Arg	Gly	Thr	Ile
GCT Ala		TCG Ser		GTG val		AGT Ser		ATT Ile	

GCT Ala		CCG Pro		GTG Val		GGG Gly		ATC Ile	
GCT Ala		TCC Ser		GTA Val		GGA Gly		ATT Ile	
GGA Gly		TCC Ser		GTG Val		GGA Gly		ATC Ile	
GCT Ala		TCA Ser		GCA Ala		GGA Gly		ACC Thr	
GCT Ala		CCA Pro		GTG Val		GGG Gly		ACC Thr	
GCA Ala		CCT Pro		GCG Ala		GGG Gly		ATT Ile	
GCT Ala		CCT Pro		GCT Ala		GGG Gly		ATA Ile	
ACC Thr		TCT Ser		GCC Ala		AGG Arg		ACA Thr	
GGG Gly		CCA Pro		GCT Ala		GGG Gly		ATC Ile	
GCG Ala		CCT Pro		GTC Val		GGA Gly		ATT Ile	
GGA Gly		TCC Ser		GTG Val		GGA Gly		ATC Ile	
GGT Gly		TCT Ser		GTA Val		GGG Gly		ATC Ile	
ACG Thr		TCC Ser		GCG Ala		GGA Gly		ATT Ile	
GCT Ala		TCG Ser		GTT Val		GGG Gly		ATC Ile	
GCG Ala		CCA Pro		GCT Ala		GGA Gly		ACT Thr	
GCT Ala		CCT Pro		GCA Ala		GGG Gly		ATC Ile	
GGG Gly		TCA Ser		GTG Val		GGG Gly		ATC Ile	
GGT Gly		CCG Pro		GCT Ala		GGA Gly		ATT Ile	
AGT Ser		TCC Ser		GTC Val		GGG Gly		ATA Ile	
GCT Ala		TCC Ser		GCT Ala		GGG Gly		ATA Ile	
GGA Gly		CCT Pro		GCA Ala		GGG Gly		ATC Ile	
GGT Gly		TCC Ser		GTA Val		GGA Gly		ATC Ile	
GCC Ala		TCG Ser		GCT Ala		GGA Gly		ATT Ile	
GCA Ala		CCT Pro		GCG Ala		GGG Gly		ATT Ile	
GCG Ala		TCG Ser		GCT Ala		GGA Gly		ATT Ile	
GCT Ala		TCT Ser		GTG Val		GGG Gly		ATC Ile	
GGT Gly		TCG Ser		GTG Val		GGA Gly		ATA Ile	
GGT Gly		CCT Pro		GCC Ala		GGG Gly		ATT Ile	
GGTGly		TCG Ser		GTG Val		GGG Gly		ATA Ile	
AGC Ser		TCG Ser		GTA Val		GGA Gly		ATA Ile	
GCC Ala		TCC Ser		GCA Ala		AGG Arg		ACC Thr	
GCT Ala		CCG Pro		GCC Ala		GGA Gly		ATA Ile	
Ala 55%	Gly 33%	Ser 60%	Pro 40%	Ala 51%	Val 49%	Arg 6%	Gly 94%	Thr 15%	Ile 85%

While the first three positions showed a more or less random choice of amino acids, there is a strong bias towards MCF's glycine and isoleucine in the fourth and fifth positions, suggesting that these two amino acids are the determinants for the different tropisms of

5 SL3-2 and MCF 247 viruses Random occurrence of codons confirmed that it is the protein sequence that is important for infection of human cells A few isolated colonies contained amino acids other than those found in either SL3-2 or MCF 247 These probably result from inaccurate primer synthesis in primers used to create the library (the oligonucleotides do

not have an equal distribution of the four or two nucleotides during incorporation of the NNK sequence of the primer)

Example 4 Mutation studies

- 5 To confirm the results of the library study, four mutants were constructed, each containing a different combination of amino acids in the VR3 fourth and fifth non-homologous positions. The titers of these constructs were measured on murine NIH 3T3 and human TE 671 cells. The results are presented in table 6

10 Table 6 Titer of SL3-2 VR3 4th and 5th non-homologous position mutants

Mutated amino acids in 1 st and 2 nd positions	Titer (cfu/mL) on TE 671 cells	Titer (cfu/mL) on NIH 3T3 cells
RT (wild type)	4.75	5×10^6
RI	2.25×10^1	3.25×10^6
GT	1.5×10^3	2×10^6
GI	4.5×10^5	2×10^6

All of the constructs have similar titers on murine cells suggesting correct expression and function of the envelopes. In agreement with the results of the library study, substituting both arginine and threonine of the SL3-2 VR3 with glycine and isoleucine of MCF 247

- 15 elevates the titer on human cells by a factor of around 100 000. Single substitutions yield envelopes with intermediate titers

Substitution of either arginine or threonine yields envelopes with intermediate titers as shown in table 6, although substitutions of arginine had a more profound effect on tropism

- 20 We conclude that a glycine to arginine substitution in SL3-2 is the major determinant for limited tropism of this virus and that isoleucine to threonine has only a secondary effect

Example 4 Alignment of MLV envelopes

Alignment of MLV envelopes shows almost perfect homology in this region between

- 25 polytropic, xenotropic and amphotropic sequences. It is possible that this segment plays an important role in mediating fusion by Env. Since the receptor for polytropic/xenotropic viruses differ from those for amphotropic viruses, the function of the VR3 segment might be independent of the receptor binding. This idea is confirmed by the fact that this region is outside the receptor binding domain of amphotropic and ecotropic envelopes (Battini *et al*, 1992), (Heard *et al*, 1991)
- 30

- In apparent disagreement, the entire N-terminal segment of polytropic and xenotropic envelopes including the (polytropic or xenotropic but not amphotropic) proline rich region
- 35 is necessary for envelope function (Battini *et al*, 1992), (Battini *et al*, 1995). Polytropic and xenotropic N-terminal fragments of SU cannot confer superinfection resistance unless

they also include the proline rich region, but the proline rich region neither binds the receptor on its own nor changes the tropism of the amphotropic envelope if it replaces amphotropic PRR

- 5 These data suggest that the binding domain of polytropic and xenotropic envelopes are longer than that of ecotropic or amphotropic envelopes and also include the proline rich region. It is also possible that VR3 and proline rich region of polytropic and xenotropic envelopes are important in correct folding of the receptor binding domain and are not directly involved in binding the receptor

- 10 The mutations in SL3-2 only affect the infection of human cells, while the mouse cells are infected effectively

Example 5 The mini-virus system

- 15 Mini-virus system consists of a vector, expressing a marker gene and envelope, and of a complementing cell line, designated a semi-packaging cell line, expressing Gag and Pol proteins. Expression of envelope is achieved by inserting an IRES element from EMCV between the marker and *env* genes. The major difference between the mini-virus system and conventional vector systems is that in mini-virus envelope is expressed from the transfer vector whereas in other systems, it is expressed from the packaging cells

- 20 Expressing envelope from the transfer vector instead of the packaging cells has two major advantages. First superinfection resistance in packaging cell line is avoided. In traditional packaging cell lines, envelope is expressed constitutively, resulting in superinfection resistance. Hence a virus using the same receptor cannot infect these cells. This makes it impossible for a vector to spread in a culture of packaging cells. In the mini-virus system this is avoided by expressing envelope from the vector. The mini-virus is thus replication competent in the semi-packaging cell line (that expresses Gag and Pol), but not in other cells. Secondly, since the viruses carry the *env* gene, the phenotype of the envelope protein can be directly linked to the genotype of a particular *env* gene, just as studies with wild type viruses. See figure 5

- 35 These properties, and the fact that infected cells can be easily selected using the marker gene, makes this system very suitable for studying envelope proteins. Random mutational analysis can be done, in which envelopes with particular characteristics can be selected from a large pool and their primary structure deduced from the sequence of integrated vector in the cellular genome. The relatively small size of mini-virus makes creation of random mutations in specific parts of *env* possible by PCR based methods (Jespersen *et al* , 1997). Alternatively, evolutionary studies of envelope can be done, in which selection of the marker gene can drive *in vitro* evolution of the envelope protein

- 40 The vector employed in this study Neo-env-mo (see figure 6) is based on Akv MLV (Van Beveren *et al* , 1985) and contains IRES element derived from ECMV (Morgan *et al* , 1992) and the *neomycin phosphotransferase II* (Beck *et al* , 1982) (*neo*) as the marker gene

The *neo* gene confers upon mammalian cells resistance to the aminoglycoside antibiotic G418, which otherwise blocks protein synthesis

The overlap extension method (Jespersen *et al* , 1997) was used to create the mutants and
5 the randomised libraries This method benefits from the fact that introduction of
alternative or random nucleotides into ends of a PCR-amplified DNA fragment pool is
possible if the primers used are designed accordingly To introduce such randomisation
inside a vector, two fragments, one of which contains the randomised sequence, are
synthesised using PCR The fragments are designed so that they span the whole vector
10 when combined and at the same time share an overlapping sequence at their ends The
two fragments can act as primers for each other in a subsequent PCR by virtue of the
overlapping sequence The second PCR thus reproduces the vector that contains the
randomized sequence To increase the yield, end primers were added to amplify the
overlap extension product as it is formed See figure 6

15

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Claims

- 1 A purified retroviral envelope polypeptide, capable of mediating infection of a cell by use of the polytropic/xenotropic receptor encoded by the *Rmc1* locus of the NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of a cell by use of a human
- 5 polytropic/xenotropic receptor encoded by the human RMC1 locus
- 2 A purified retroviral envelope polypeptide, capable of mediating infection of a cell derived from *Mus musculus* by use of the polytropic/xenotropic receptor encoded by the *Rmc1* locus isolated from a NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of a human cell solely expressing a human polytropic/xenotropic
- 10 receptor encoded by the human RMC1 locus
- 3 A purified murine retroviral envelope polypeptide, capable of mediating infection of a cell derived from *Mus musculus* using the polytropic/xenotropic receptor encoded by the *Rmc1* locus from a NIH Swiss inbred NFS/N mouse for entry, and unable of mediating infection of a human cell comprising a human polytropic/xenotropic receptor encoded by
- 15 the human RMC1 locus
- 4 A purified retroviral envelope polypeptide comprising an amino acid sequence which is at least 94% identical to the amino acid sequence shown in SEQ ID NO 2, or a fragment of said amino acid sequence that is at least 94% identical to the sequence shown in SEQ ID NO 2
- 20 5 A purified retroviral envelope polypeptide according to claim 4, wherein said polypeptide includes at least one substitution in the VR3 region
- 6 A purified retroviral envelope polypeptide according to claim 5, wherein said mutation is at position 212 in SEQ ID NO 2
- 7 A purified retroviral envelope polypeptide according to claim 5 or 6, wherein said at
- 25 least one substitution alters the host tropism of a virus or an infectious particle comprising said polypeptide
- 8 A purified retroviral envelope polypeptide according to any of claims 5-7, wherein said purified polypeptide is a murine retroviral envelope polypeptide capable of mediating infection of a human cell
- 30 9 A purified retroviral envelope polypeptide according to any of claims 5-8 capable of mediating a higher infectivity in human cells than MCF-247, MCF-13 and X-MLV (NZB) viruses
- 10 A purified retroviral envelope polypeptide according to any of claims 1-9, further comprising an inserted non-viral sequence capable of redirecting the target cell specificity,
- 35 by the resultant chimeric envelope

- 11 A purified retroviral envelope polypeptide according to claim 10, wherein the chimeric envelope further contains secondary mutations, enabling activation of the fusogenic properties of said chimeric envelope, by binding to the receptor target
- 12 A purified retroviral envelope polypeptide according to any of claims 10 and 11,
5 wherein said inserted sequence is a single chain antibody
- 13 A purified retroviral envelope polypeptide according to any of claims 1-12, further comprising a chemical modification of said envelope
- 14 A purified retroviral envelope polypeptide according to claim 13, wherein said chemical modification enhances and/or alters the host tropism
- 10 15 A recombinant mammalian cell displaying an envelope polypeptide according to any of claims 1-14
- 16 An isolated nucleic acid sequence encoding any of the envelope polypeptides according to any of claims 4-9
- 17 An isolated nucleic acid sequence as shown in SEQ ID NO 1
- 15 18 A recombinant mammalian expression vector comprising a purified envelope polypeptide according to claims 1-4 and/or 10-14
- 19 A recombinant mammalian expression vector comprising a purified envelope polypeptide according to claims 5-9 and/or 10-14
- 20 20 A replication competent retrovirus comprising a purified envelope polypeptide according to any of claims 1-4 and/or 10-14
- 21 A replication competent retrovirus, comprising a purified envelope polypeptide according to any of claims 5-9 and/or 10-14
- 22 A vector according to 20 and 21 further comprising a heterologous translation cassette
- 25 23 A vector according to claim 22, wherein said heterologous translation cassette consists of an IRES-gene element
- 24 A retroviral expression vector comprising a purified envelope polypeptide according to claims 1-4 and/or 10-14
- 25 A retroviral expression vector comprising a purified envelope polypeptide according to
30 claims 5-9 and/or 10-14
- 26 A vector according to claim 18 or 19 or 24-25, further comprising at least one heterologous gene to be expressed

- 27 A vector according to claim 26, wherein expression of the envelope is directed by a IRES-element
- 28 A packaging cell construct comprising a recombinant mammalian expression vector comprising a nucleic acid coding for a purified envelope polypeptide according to any of
5 claims 1-14, and a non-viral or viral promoter and poly-adenylation signals
- 29 Use of a vector according to any of claims 18-19 and/or 28 for the generation of a packaging cell
- 30 Use of a vector according to any of claims 24-27, for expression in a cell constitutively expressing the gag/pol genes of simple retroviruses
- 10 31 Use of a packaging cell according to any of claims 28-30 for the preparation of a composition for the modification of a cell
- 32 Use of a vector according to claims 22 and/or 23, for the preparation of a composition for the modification of a cell
- 33 Use of a virus or vector according to any of claims 18-27 for gene discovery by
15 infection of a new-born rodent
- 34 Use of a virus or vector according to claim 33, wherein said rodent constitutively express the gag/pol genes of simple retroviruses
- 35 Use of a virus or vector according to claim 33, wherein said rodent express the gag/pol genes of simple retroviruses in a tissue specific manner
- 20 36 Use of a virus or vector according to claim 33, wherein said rodent expression of the gag/pol genes of simple retroviruses is developmentally regulated
- 37 Use of a virus or vector according to claim 33, wherein said rodent expresses the gag/pol genes of gamma retroviruses tissue specifically and in a developmentally regulated manner
- 25 38 A method for gene discovery comprising
- a) using a virus or vector according to any of claims 18-27
 - b) infecting a new-born rodent with said virus or vector
 - c) inducing a tumour by means of said virus or vector
 - d) isolating said tumour in said rodent
- 30 e) identifying a gene involved in the oncogenesis by cloning the integration site of said virus or vector in said tumour

39 A method according to claim 38 for gene discovery of a cancer related gene

40 Use of any of the envelope polypeptides according to claims 1-4 and/or 10-14 or vectors comprising said polypeptides in a bio-safety level 1/PS I/SI laboratory animal facilities or equivalents thereof

	277	293
SL3-2	(199)	ASKAWGLRLYRSTRTDP
MCF-247	(199)	GPKVWGLRLYRSTGTDP
MCF CI-3	(199)	GPKVWGLRLYRSTGTDP
Ampho-MCF	(199)	GPKVWGLRLYQSTGTDP
ERV-1	(199)	GPKVWGLRLYRSTGTDP
Endogenous from 129 GIX+ mice	(199)	GPKVWGLRLYRSTGTDP
Friend MCF #2	(199)	GPKVWGLRLYRSTGTDP
Friend MCF	(199)	GPKVWGLRLYRSTGTDP
Friend SFV	(199)	GPKVWGLRLYRSTGTDP
Invitro MCF	(199)	GPKVWGLRLYRSTGTDP
MCF 1223	(199)	GPKVWGLRLYRPTGTDP
MLV DBA/2	(199)	GPKVWGLRLYRSTGTDP
MCF (Broscius)	(199)	GPKVWGLRLYQSTGTDP
Mo-MCF	(199)	GPKVWGLRLYRSTGTDP
Ns-6 (186) MCF	(199)	GPKVWGLRLYRSTGTDP
Rauscher sfv	(199)	GPKVWGLRLYRSTGTDP
R-XC-	(199)	GPKVWGLRLYRSTGTDP
MCF (Ter-Grigorov)	(199)	GPKVWGLRLYRSTGTDP
AKV	(252)	TGHWWGLRLYVS-GHDP
Friend	(237)	IGHYWGLRLYVS-GODP
Moloney	(234)	TGHYWGLRLYVS-GODP
SL3-3	(252)	TGHWWGLRLYVS-GHDP
Friend fass	(237)	TGHYWGLRLYVS-GRDP
10A1	(209)	GPKSWGLRLYRT-GTDP
4070A	(209)	GPKSWGLRLYRT-GTDP
Xeno CWM-S-5X	(202)	APKVWGLRLYRSTGADP
DG-75 Xeno	(202)	APKVWGLRLYRSTGADP
Xeno NZB-9-1	(202)	APKVWGLRLYRSTGADP
Xeno Bxv-1-related	(202)	APKVWGLRLYRSTGADP
Xeno R-MCF-1	(202)	GPKVWGLRLYRSTGTDP
Consensus	(277)	GPKVWGLRLYRSTGTDP

Fig. 1

2/6

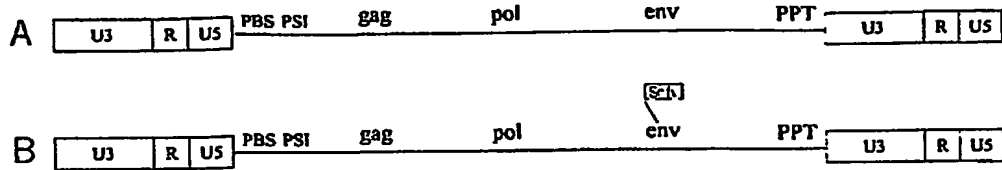
		Section 1									
		(1)	10	20	30	40	50	60	70	80	85
SL3-2	(1)	MEGPAFSKPLKDKINPWGELIVLGLTMRARVSVONDSPHOVFNVTWRVTNLMTGOTANATSLIGT									
MCF-247	(1)	MEGPAFSKPLKDKINPWGELIVLGLTMRARVSVONDSPHOVFNVTWRVTNLMTGOTANATSLIGT									
Leader											
		Section 2									
		(66)	66	80	90	100	110	120	130	140	150
SL3-2	(66)	MTDAFPRLYEDCDLIGDDWDETGLGCRTPGGGRKRARIFDFYVCPGHTVLACGGGPRGYCGKWG									
MCF-247	(66)	MTDAFPRLYEDCDLIGDDWDETGLGCRTPGGGRKRARIFDFYVCPGHTVLACGGGPRGYCGKWG									
VRA											
		Section 3									
		(131)	131	140	150	160	170	180	190	200	195
SL3-2	(131)	CETTGOAYWKPFSSSWDLISLKRGNTPKGGGPCYDSSVSSSAQGATPGGRCNRLVLEFTDAGKRA									
MCF-247	(131)	CETTGOAYWKPFSSSWDLISLKRGNTPKGGGPCYDSSVSSSAQGATPGGRCNRLVLEFTDAGKRA									
VRB											
		Section 4									
		(196)	196	210	220	230	240	250	260	270	260
SL3-2	(196)	SWDASKAWGLRLYRSTRTRDEVRESLTROVLNIGPRVFIQGNPVITBOLPPSRVQIMLPREPOB									
MCF-247	(196)	SWDASKAWGLRLYRSTRTRDEVRESLTROVLNIGPRVFIQGNPVITBOLPPSRVQIMLPREPOB									
VR3											
		Section 5									
		(261)	261	270	280	290	300	310	320	330	326
SL3-2	(261)	PPFGAAS TVBETAPPSQOPGTGDRLLNLVKGAYQALNLTSPDKTQECWLCVAGPPYYEGVAVLG									
MCF-247	(261)	PPFGAAS TVBETAPPSQOPGTGDRLLNLVKGAYQALNLTSPDKTQECWLCVAGPPYYEGVAVLG									
		Section 6									
		(326)	326	340	350	360	370	380	390	400	390
SL3-2	(326)	TYSNHTSAPANC SVASQHKLTLSVITGQGLCYGAVPKTHQALONTTQRTSNGSYLAAPACTIWS									
MCF-247	(326)	TYSNHTSAPANC SVASQHKLTLSVITGQGLCYGAVPKTHQALONTTQRTSNGSYLAAPACTIWS									
		Section 7									
		(391)	391	400	410	420	430	440	450	460	455
SL3-2	(391)	QNTGLTPCESTTHLDTTDYCVLVELWPKVTVYHSPGYVXGOFEEKTKYKREPVS LTLALLGGLT									
MCF-247	(391)	QNTGLTPCESTTHLDTTDYCVLVELWPKVTVYHSPGYVXGOFEEKTKYKREPVS LTLALLGGLT									
		Section 8									
		(456)	456	470	480	490	500	510	520	530	520
SL3-2	(456)	MGGIAAGVGTGTTALVATQOQFOOLQAAMQDOLKEVEKSTTNLERSLTSLSEVVLONRRGLDLLFL									
MCF-247	(456)	MGGIAAGVGTGTTALVATQOQFOOLQAAMQDOLKEVEKSTTNLERSLTSLSEVVLONRRGLDLLFL									
		Section 9									
		(521)	521	530	540	550	560	570	580	590	585
SL3-2	(521)	KEGGLCAALKEECCFYADHTGLVRDSMAKLRERLSORQKLFESQOQWFEGLENKSPWFTTLISTI									
MCF-247	(521)	KEGGLCAALKEECCFYADHTGLVRDSMAKLRERLSORQKLFESQOQWFEGLENKSPWFTTLISTI									
		Section 10									
		(586)	586	600	610	620	630	640	650	660	641
SL3-2	(586)	MGPLIILLILLILLEGPCILNHLVQFQKDRSVVQALVLTQOYHOLKSTEDCESRE--									
MCF-247	(586)	MGPLIILLILLILLEGPCILNHLVQFQKDRSVVQALVLTQOYHOLKSTEDCESRE--									

Fig. 2

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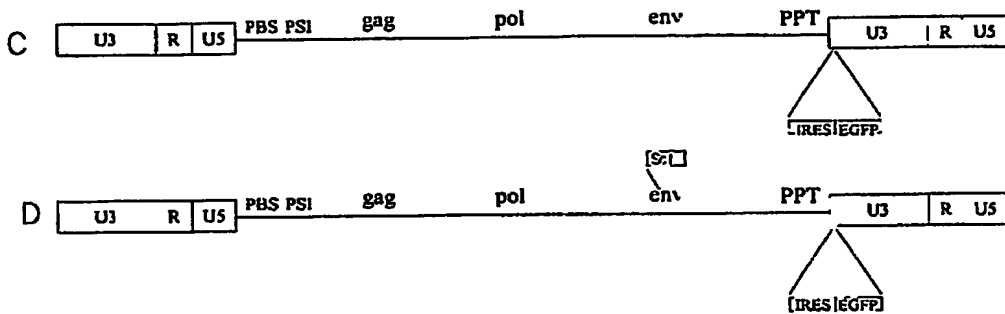
3/6

Replication competent virus

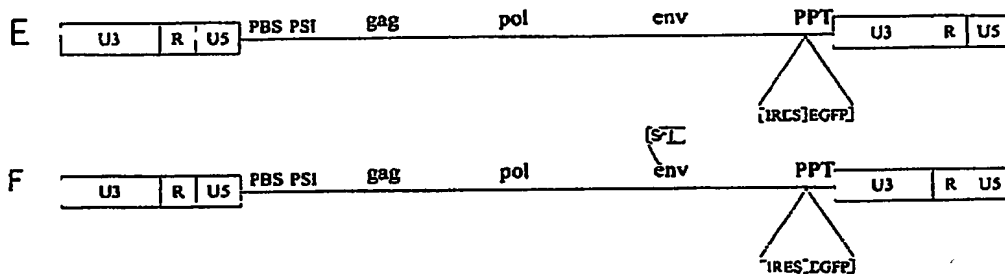


Replication competent vector

U3 type of maxivirus



3Prime untranslated type of maxivirus



A retroviral expresion vector containing envelope (Minivirus)

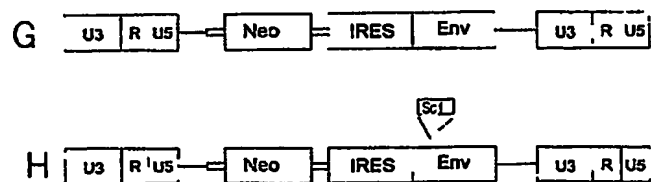


Fig. 3

4/6

Generation of DNA library

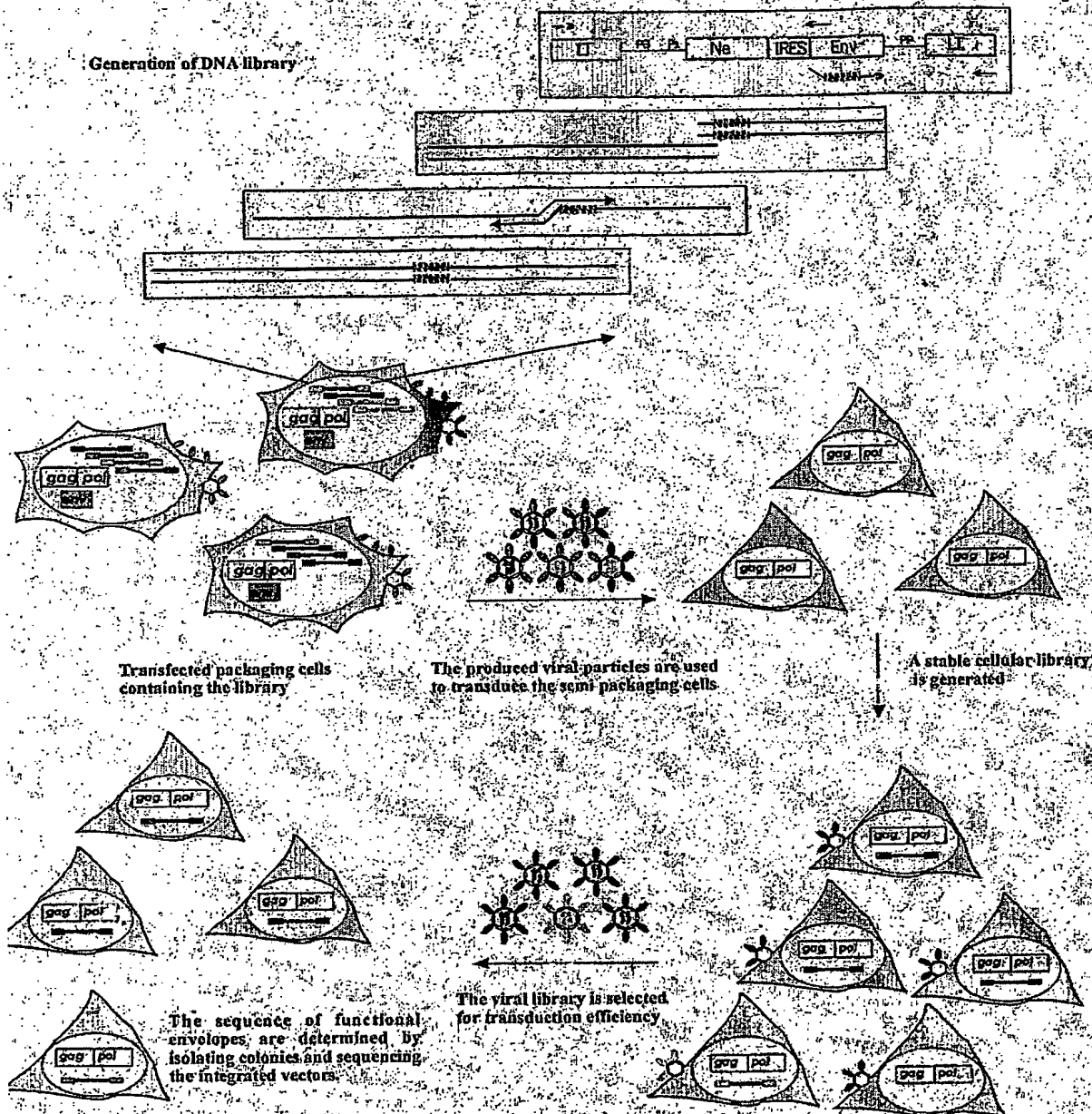


Fig. 4

5/6

Modtaget

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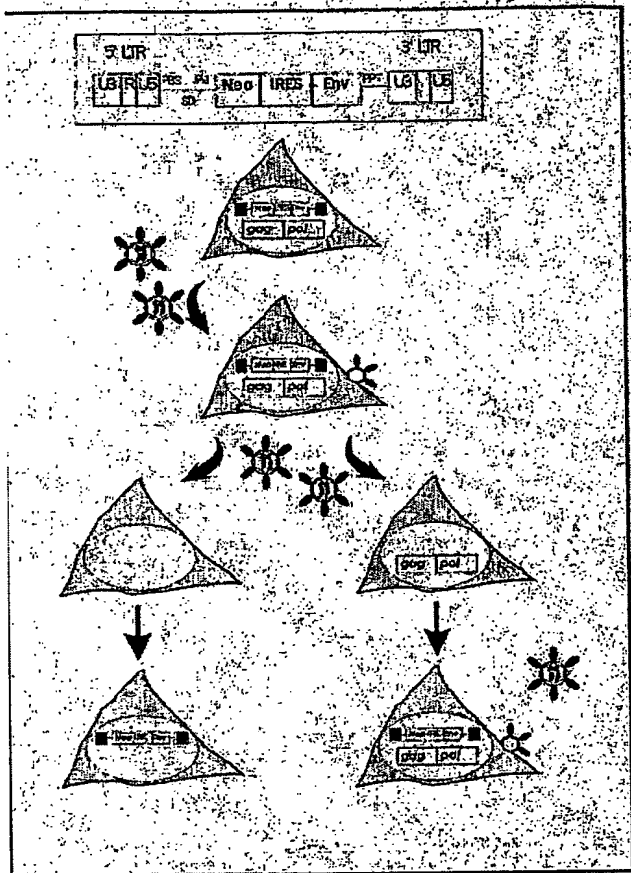


Fig. 5

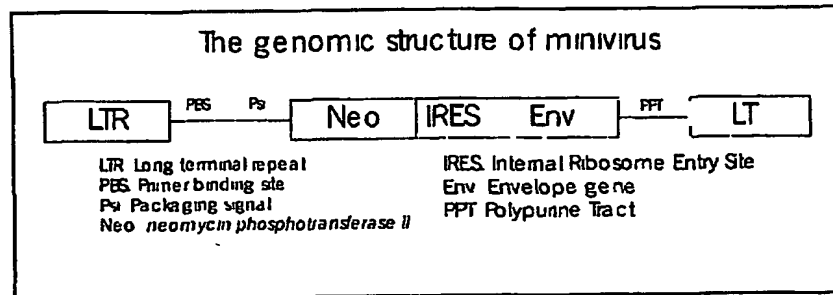


Fig. 6

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